IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
BROWNING et al.	)	Group Art Unit: 1644
	)	
Serial No.: 09/911,777	)	Examiner: Haddad, Maher M.
	)	
Filed: July 24, 2001	)	
	)	
For: BAFF, INHIBITORS THEREOF AND THEIR USE IN THE MODULATION OF B-CELL RESPONSE	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION OF SUSAN KALLED UNDER 37 C.F.R. § 1.132

I, Susan Kalled, declare:

1. I am a Principal Scientist in Molecular and Cellular Biology at Biogen Idec and have been employed at Biogen Idec (and its predecessor company, Biogen) in various scientific capacities since 1995. I received my Ph.D. in Immunology from Tufts University in 1991, and my B.A. in Microbiology from the University of New Hampshire in 1983. Most recently, I have been the Project Leader at Biogen Idec for the BAFF Program, and have authored or co-authored four review articles on the subject of BAFF and other TNF family members.

2. I have read and understood application Serial No. 09/911,777, including the claims as amended in the response filed with this Declaration. The claims, as amended, are drawn to methods of inhibiting B-cell growth, immunoglobulin production, or both. The claimed methods involve administering to an animal a therapeutically effective amount of an anti-BAFF antibody that specifically recognizes human or murine BAFF.

3. We have evaluated treatment of BAFF transgenic (BAFF Tg) mice with a soluble form of a BAFF receptor, BCMA (B cell maturation antigen).

4. BAFF Tg mice expressing full-length murine BAFF under the control of liver specific regulatory sequences were generated as described in Mackay et al. (1999) *J. Exp. Med.*, 190(11):1697-710. BCMA-Fc was prepared as described in Thompson et al. (2000) *J. Exp. Med.*, 192(1):129-35.

5. Six-month-old BAFF Tg mice and non-Tg littermate controls received intraperitoneal injections of PBS, 400 µg of BCMA-Fc, or 400 µg of polyclonal human IgG (hIg) once a week for five weeks.

6. We monitored proteinuria each week to assess renal function, and upon sacrifice, analyzed spleen weight and total serum Ig titers. The effect on splenomegaly was determined by examining spleen weights of treated and control mice. Splenomegaly and hypergammaglobulinemia were reduced upon treatment with BCMA-Fc as compared to a control. As shown in Table 1, the geometric mean of total serum Ig was reduced four-fold in BAFF Tg mice treated with BCMA-Fc, a significant reduction ($p=0.02$) when compared with BAFF Tg mice treated with control antibody.

These results indicate that immunoglobulin production is inhibited following administration of the soluble BAFF receptor.

Table 1.

Mouse/Treatment		Total Serum Ig (mg/ml)
BAFF Tg/hIgG		
802C06		6.7
816B82		4.7
816D20		10.5
816E34		4.4
823C48		13
823C69		9.7
	Geo Mean	7.5
BAFF Tg/BCMA-Fc		
802C05		2
802C07		0.7
802C09		1.4
823C39		2.2
823C41		1.5
823C45		8
	Geo Mean	1.9
Non-Tg/hIgG		
823B64		3.9
823B66		2.5
823B94		5.6
823B98		2.8
	Geo Mean	3.5

7. We further assessed the effect of BCMA-Fc on the development of nephritis in the BAFF Tg mice, which involves immune complex deposition. Proteinuria

scores were determined prior to, during and after treatment. At the end of the 5-week treatment period, hIgG-treated mice showed no significant improvement over the pre-treatment score when compared to PBS-treated controls. In contrast, BCMA-Fc treated mice exhibited an average 50% improvement in proteinuria score at the end of the 5-week treatment period (Fig. 1). These results further demonstrate that immunoglobulin production is inhibited following administration of a soluble BAFF receptor.

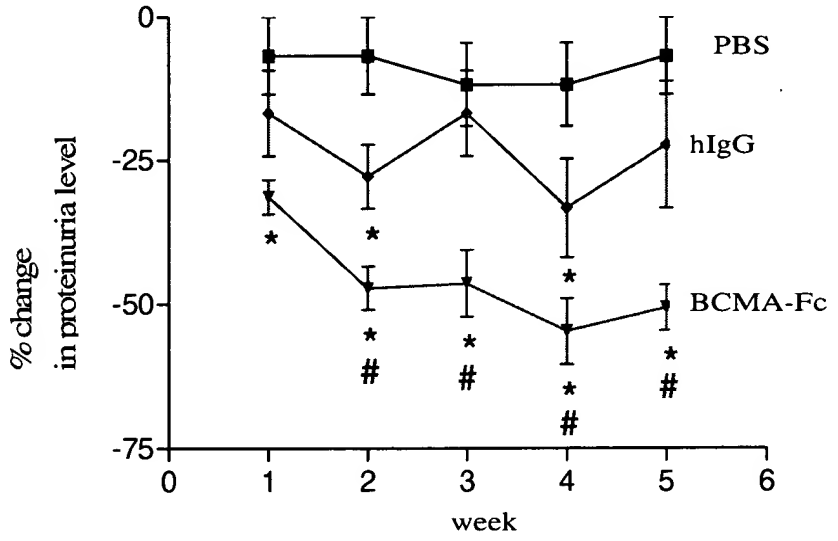

Figure 1

8. We also assessed the effect of BCMA-Fc on B cell numbers in the BAFF Tg mice. Single cell suspensions from spleens were prepared and analyzed by flow cytometry as described in Mackay et al. (1999) *J. Exp. Med.*, 190(11):1697-710. Splenocytes were stained with a fluorochrome-conjugated anti-B220 mAb to detect B lymphocytes as described in Thompson et al. (2001) Science, 293:2108-2111.

9. BAFF Tg mice treated with control antibody (BAFF Tg/hIgG) had elevated numbers of B lymphocytes, including marginal zone (MZ, IgM^{bright}/ IgD^{-}/ $CD21^{bright}$) (Fig. 2, upper panel) and mature (IgM^{low} / IgD^{+}/ $CD21^{int}$) (Fig. 2, lower panel) B cells. Following treatment with BCMA-Fc (Tg BCMA), splenic levels of MZ B cells were reduced 10-fold (Fig. 2, upper panel) and mature B cells were reduced 11-fold (Fig. 2, lower panel) compared to BAFF Tg mice treated with control antibody (Tg hIgG). These results indicate that B cell growth is inhibited following administration of a soluble BAFF receptor.

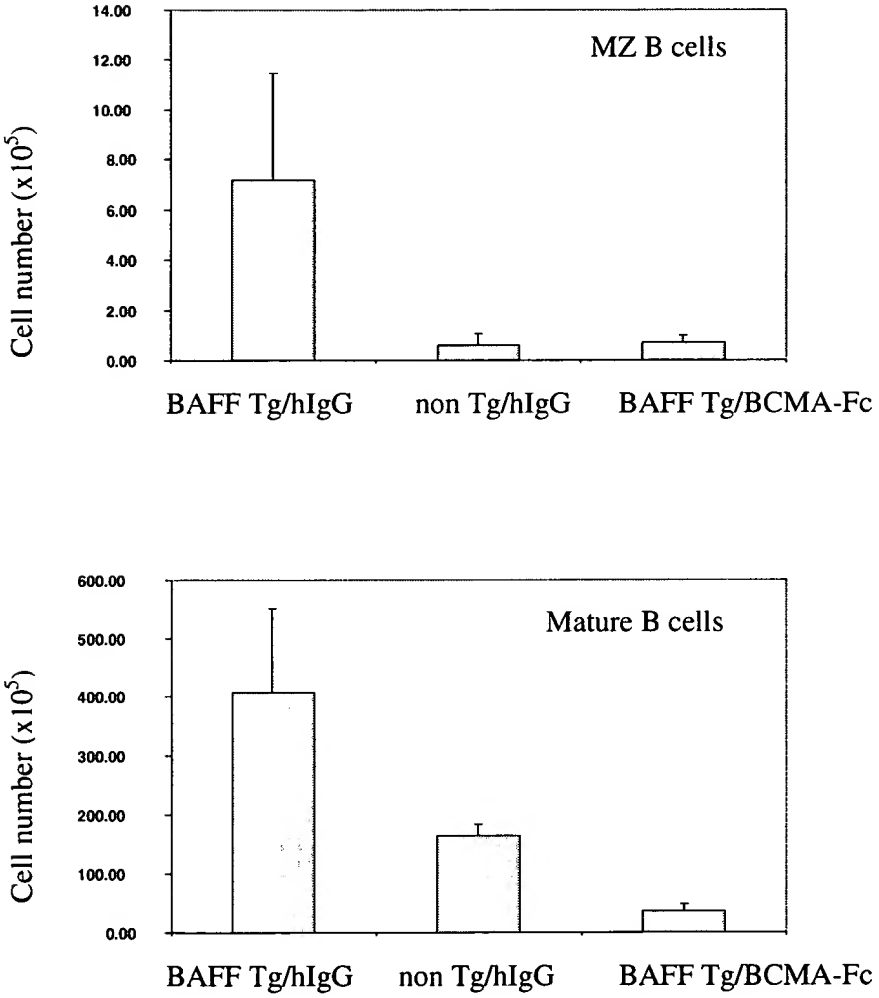
MZ B cells
Cell number (x10^5)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
BAFF Tg/hIgG
non Tg/hIgG
BAFF Tg/BCMA-Fc
Mature B cells
Cell number (x10^5)
600.00
500.00
400.00
300.00
200.00
100.00
0.00
BAFF Tg/hIgG
non Tg/hIgG
BAFF Tg/BCMA-Fc

Figure 2

10. The above results demonstrate that sequestration of BAFF in a mouse model of Sjögren's syndrome leads to a reduction in total serum Ig, splenolomegaly, and the numbers of MZ and mature B cells.

11. Based on these results, I expect that administration of soluble forms of other BAFF receptors, such as BAFF-R and TACI, as well as anti-BAFF antibodies, will likewise result in a reduction in total serum Ig, splenolomegaly, and the numbers of MZ and mature B cells.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Jan. 8, 2004

By: Susan Kalled
Susan Kalled, Ph.D.

Effects of LymphoStat-B, a BLyS Antagonist, when Administered Intravenously to Cynomolgus Monkeys.

Category: 26 SLE—animal models

Wendy B. G. Halpern[1], Patrick Lappin[2], Thomas Zanardi[2], David M. Hilbert[1], Paul A. Moore[1], Vivian R. Albert[1], Kevin P. Baker[1]. [1]Human Genome Sciences Inc., Rockville, MD; [2]Charles River Laboratories, Sparks, NV

Presentation Number: 1537

Poster Board Number: 380

Purpose: This study was conducted to evaluate the tolerability and effects of LymphoStat-B administered over 6 months to cynomolgus monkeys. LymphoStat-B is a fully-human IgG_1 lambda antibody directed against B-lymphocyte stimulator (BLyS). BLyS is a TNF family member that supports B-lymphocyte maturation and survival and has been implicated in the pathogenesis of several autoimmune diseases. LymphoStat-B was developed to antagonize the activity of BLyS in autoimmune disease, where undesirable effects of B-lymphocyte activity may cause or contribute to disease. LymphoStat-B binds specifically and with high affinity to recombinant BLyS protein from both humans and cynomolgus monkeys, and neutralizes their bioactivity *in vitro*.

Methods: LymphoStat-B was administered intravenously every other week to 16 monkeys per group at 5, 15 or 50 mg/kg/dose. A vehicle control was administered to 12 monkeys. Pharmacodynamic study endpoints included immunophenotyping of peripheral blood and tissues (spleen and lymph node), as well as standard clinical and anatomic pathology. Pathology endpoints were evaluated after 3 and 6 months of treatment, and after an 8-month treatment free (recovery) period.

Results: LymphoStat-B was well tolerated when administered intravenously to cynomolgus monkeys at doses up to 50 mg/kg for as long as 26 weeks, with no treatment-related infections identified. As detected by flow cytometric methods, monkeys exposed to LymphoStat-B had significant decreases in peripheral blood $CD20^+$ lymphocytes (B-cells) and $CD20^+/CD21^+$ lymphocytes (mature B-cells) after 13 weeks of exposure, with concomitant decreases in spleen and lymph node B-lymphocyte representation (both $CD20^+$ and $CD20^+/CD21^+$ cells). In contrast, neither $CD3^+$ T-lymphocytes nor $CD3^-/CD14^+$ monocytes were affected by LymphoStat-B. Microscopically, monkeys treated with LymphoStat-B had mild to marked decreases in the number and size of lymphoid follicles in the white pulp of the spleen. In addition, decreased spleen weights were evident after 26 weeks of exposure in LymphoStat-B treated monkeys. Overall there was a general correlation between peripheral blood B-lymphocytes, tissue B-lymphocyte representation, spleen weights and histologic findings. Total lymphocyte counts were similar in all groups throughout the study. In this study LymphoStat-B administration did not clearly affect globulins, albumin to globulin ratio, or immunoglobulin subclasses. All findings were generally reversible within the 8 month recovery period.

Conclusions: These data confirm the specific pharmacologic activity of LymphoStat-B in reducing B-lymphocytes in the cynomolgus monkey. Furthermore, the nonclinical safety profile of LymphoStat-B in monkeys supports its clinical development as a potential therapeutic for the treatment of autoimmune disease.

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	Presentation	Authors	Session
1. ☐	922. Safety, Pharmacokinetic and Pharmacodynamic Results of a Phase 1 Single and Double Dose-Escalation Study of LymphoStat-B (Human Monoclonal Antibody to BLyS) in SLE Patients (Board 317)	R. Furie[1], W. Stohl[2], E. Ginzler[3], M. Becker[4], N. Mishra[5], W. Chatham[6], Joan T. Merrill[7], A. Weinstein[8], W. J. McCune[9], J. Zhong[10], W. Freimuth[10], and the LymphoStat-B Study Group. [1]North Shore Univ Hosp, Manhasset, NY; [2]USC, Los Angeles, CA; [3]SUNY Downstate, Brooklyn, NY; [4]U Chicago, Chicago, IL; [5]Wake Forest U, Winston-Salem, NC; [6]UAB, Birmingham, AL; [7]OMRF, Oklahoma City, OK; [8]Wash Hosp Ctr, Washington, DC; [9]U Michigan, Ann Arbor, MI; [10]Human Genome Sciences, Rockville, MD	**ACR/ARHP Poster Session B** SLE Treatment—Biologic Agents Sunday, 8:00 a.m. - 4:00 p.m. Convention Center - Hall D - E
2. ☐	1537. Effects of LymphoStat-B, a BLyS Antagonist, when Administered Intravenously to Cynomolgus Monkeys. (Board 380)	Wendy B. G. Halpern[1], Patrick Lappin[2], Thomas Zanardi[2], David M. Hilbert[1], Paul A. Moore[1], Vivian R. Albert[1], Kevin P. Baker[1]. [1]Human Genome Sciences Inc., Rockville, MD; [2]Charles River Laboratories, Sparks, NV	**ACR/ARHP Poster Session C** SLE—Animal Models II: B-Cells/Pathogenesis Monday, 8:00 a.m. - 4:00 p.m. Convention Center - Hall D - E

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Safety, Pharmacokinetic and Pharmacodynamic Results of a Phase 1 Single and Double Dose-Escalation Study of LymphoStat-B (Human Monoclonal Antibody to BLyS) in SLE Patients

Category: 24 SLE—treatment: developments in the treatment of SLE

R. Furie[1], W. Stohl[2], E. Ginzler[3], M. Becker[4], N. Mishra[5], W. Chatham[6], Joan T. Merrill[7], A. Weinstein[8], W. J. McCune[9], J. Zhong[10], W. Freimuth[10], and the LymphoStat-B Study Group. [1]North Shore Univ Hosp, Manhasset, NY; [2]USC, Los Angeles, CA; [3]SUNY Downstate, Brooklyn, NY; [4]U Chicago, Chicago, IL; [5]Wake Forest U, Winston-Salem, NC; [6]UAB, Birmingham, AL; [7]OMRF, Oklahoma City, OK; [8]Wash Hosp Ctr, Washington, DC; [9]U Michigan, Ann Arbor, MI; [10]Human Genome Sciences, Rockville, MD

Presentation Number: 922

Poster Board Number: 317

Purpose: LymphoStat-B is a fully human monoclonal antibody (mAb), which inhibits soluble B-Lymphocyte Stimulator (BLyS). A randomized double-blind study evaluated the safety, tolerability, immunogenicity and pharmacology (PK) of 4 different doses (1, 4, 10, 20 mg/kg) of LymphoStat-B or placebo administered as a single IV infusion or 2 infusions 21 days apart. Subjects had stable mild to moderate SLE disease activity and were on a stable standard of care SLE treatment regimen for 2 months prior to enrollment.

Methods: Patients were followed for 84-105 days for assessment of adverse events (AEs), PK and safety plus measurement of peripheral B-cell concentrations, serologies and disease activity (SELENA SLEDAI). Data from placebo subjects (n=13) in single or double dose cohorts were pooled and compared to LymphoStat-B subjects (n=57) in each of the 4 single or double dose cohorts.

Results: Study subjects were predominantly female (91%) with an average age of 41. The mean disease duration was 8.5 years with a baseline mean SELENA SLEDAI score = 2.2. LymphoStat-B was well tolerated at all doses with no study withdrawals. The overall incidence of AEs was similar between LymphoStat-B and placebo groups. There was no increased incidence of infections in the treatment group, and none of the infections reported were attributed to study agent. Six patients experienced serious adverse events with similar frequencies observed in the placebo and treatment groups. None were deemed related to study agent. Severe (grade 3 and 4) laboratory abnormalities or AEs occurred infrequently. One patient experienced an infusion reaction at the highest single dose. One patient developed neutralizing antibodies to LymphoStat-B. Pharmacokinetics of single doses were dose-proportional. Long $t_{1/2}$ = 13-17 days, slow clearance = 4.00 ± 1.56 mL/day/kg and small Vss = 68.19 ± 20.83 mL/kg are consistent with a fully human mAb. All LymphoStat-B cohorts had significant reductions of $CD20^+$ cells (12-47%) at 1 or more visits from day 42-105 compared to placebo. Reductions in anti-dsDNA or Ig levels were observed in some LymphoStat-B cohorts compared to placebo. No change in SLE disease activity was observed over this short exposure.

Conclusions: LymphoStat-B was well tolerated in SLE patients. There was a significant reduction of peripheral B-cells by LymphoStat-B consistent with its ability to bind and inhibit the biological activity of BLyS. These results support phase II trials testing for clinical benefit in patients with SLE and other autoimmune diseases.

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BAFF/BLyS Receptor 3 Binds the B Cell Survival Factor BAFF Ligand through a Discrete Surface Loop and Promotes Processing of NF-κB2

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Summary

The TNF-like ligand BAFF/BLyS is a potent survival factor for B cells. It binds three receptors: TACI, BCMA, and BR3. We show that BR3 signaling promotes processing of the transcription factor NF-κB2/p100 to p52. NF-κB2/p100 cleavage was abrogated in B cells from A/WySnJ mice possessing a mutant BR3 gene, but not in TACI or BCMA null B cells. Furthermore, wild-type mice injected with BAFF-neutralizing BR3-Fc protein showed reduced basal NF-κB2 activation. BR3-Fc treatment of NZB/WF1 mice, which develop a fatal lupus-like syndrome, inhibited NF-κB2 processing and attenuated the disease process. Since inhibiting the BR3-BAFF interaction has therapeutic ramifications, the ligand binding interface of BR3 was investigated and found to reside within a 26 residue core domain. When stabilized within a structured β-hairpin peptide, six of these residues were sufficient to confer binding to BAFF.

Introduction

Survival signals are critical for the proper development and maintenance of the immune system as they regulate the magnitude and duration of the immune response. Many cytokines, including members of the TNF family, such as CD40 ligand, have been shown to function as potent survival factors for specific lymphoid populations (Locksley et al., 2001). BAFF (also known as BLyS, TALL-1, zTNF4, THANK, and TNFS 13B) (Moore et al., 1999; Schneider et al., 1999), a recently defined member of the TNF family, is a homotrimeric type 2 transmembrane protein expressed by macrophages, monocytes, and dendritic cells. BAFF, like other members of the TNF family, also exists in a soluble form following cleavage from the cell surface by Furin-type proteases. It shares most sequence similarity with APRIL, another member of the TNF family, which is expressed by lymphoid cells and at high levels by some tumor cells (Hahne et al., 1998). BAFF is critical for the development and survival of peripheral B cells; mice lacking BAFF display an almost total loss of follicular and marginal zone B cells (Gross et al., 2001; Schiemann et al., 2001). Transgenic mice overexpressing BAFF develop autoimmune disorders characterized by B cell hyperplasia and autoantibody production including anti-DNA and rheumatoid factor. The animals eventually succumb to an immune complex-mediated, lupus-like nephritis (Gross et al., 2000; Khare et al., 2000; Mackay et al., 1999). Intriguingly, humans suffering from autoimmune syndromes, including systemic lupus erythematosus (SLE), rheumatoid arthritis, and Sjögren's syndrome, where end organ damage is primarily in the kidneys, joints, and salivary/lacrymal glands, respectively, have elevated levels of serum BAFF. Furthermore, BAFF levels correlate with disease severity, consistent with a possible role in the pathogenesis of these disabling maladies (Cheema et al., 2001; Groom et al., 2002; Zhang et al., 2001).

Of the three receptors for BAFF, only BR3 (also known as BAFF-R) is specific; the other two, TACI and BCMA, also bind the related ligand APRIL (Gross et al., 2000; Thompson et al., 2001; Yan et al., 2000, 2001a). The extracellular domain of TACI has a characteristic TNFR-like structure encompassing two cysteine-rich domains (CRDs) that are the hallmark of the TNF receptor family. These approximately 40 residue pseudorepeats have a distinct structure, typically characterized by three intrachain disulfides involving six highly conserved cysteines. BCMA is unusual in that it contains only a single canonical CRD. However, BR3 is even more divergent in that its extracellular domain is composed of only a partial CRD, containing four cysteine residues with spacing distinct from other TNFR modules characterized previously (Bodmer et al., 2002; Naismith and Sprang, 1998). Conventional members of the TNFR family utilize two CRDs for binding ligand; contacts stem primarily from analogous loops from each CRD interacting with two distinct surface patches on the ligand (reviewed in Bodmer et al., 2002). Thus, how high-affinity binding to BAFF is achieved by only a single, or partial, CRD, like that of BR3, is unclear. Recent crystal structures of BAFF reveal a trimeric TNF-like fold with several distinguishing features (Karpusas et al., 2002; Liu et al., 2002; Oren et al., 2002); however, no structure of any of the BAFF receptors has been described.

Characterization of naturally occurring mutations and knockout mice have revealed components of the B cell survival pathway potentially engaged by BAFF. Since A/WySnJ mice that possess a mutant BR3 display a profound lack of B cells, akin to BAFF null mice, it has been hypothesized that BR3 must engage a B cell survival pathway (Gross et al., 2001; Schiemann et al., 2001; Thompson et al., 2001; Yan et al., 2001a). Further, the NF-κB pathway is an attractive candidate for activation by BR3 because certain components are required for B cell survival and maintenance (Caamano et al., 1998; Franzoso et al., 1998). For example, IKKα, one of two catalytic subunits within the IκB kinase complex, is required for B cell maturation, formation of secondary

[5]Correspondence: dixit@gene.com (V.M.D.) or star@gene.com (M.A.S.)

Fig. 1a

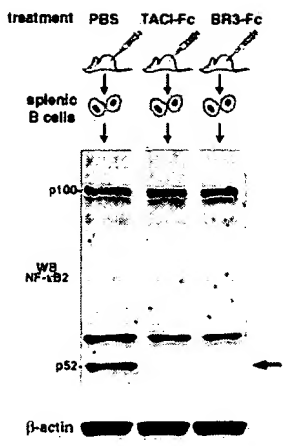


Fig. 1b

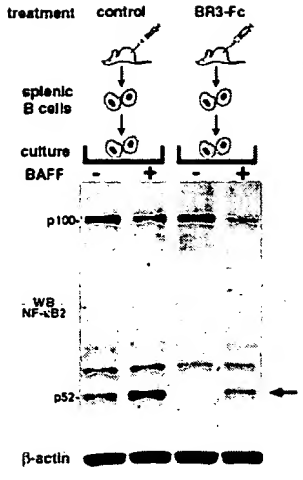


Fig. 1

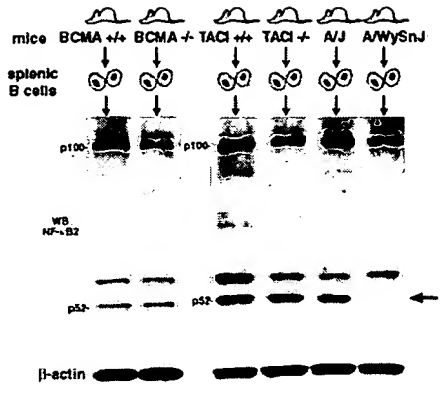


Figure 1. Involvement of Endogenous BAFF/BR3 in Basal Processing of NF-κB2 In Vivo

(A) Five C57BL/6 wild-type mice were injected intraperitoneally with PBS, 100 μg TACI-Fc, or 100 μg BR3-Fc daily for 5 days. 10 μg pooled splenic B cell lysate from each group was subjected to SDS-PAGE followed by Western blot analysis with anti-NF-κB2 or anti-β actin.

(B) Splenic B cells from PBS- or BR3-Fc-treated mice as described above were cultured with or without 1 μg/ml recombinant BAFF for 24 hr. Thereafter, 10 μg cultured B cell lysates were subjected to Western blot analysis with anti-NF-κB2 or anti-β actin.

(C) B cells were purified from pooled spleens of C57BL/6 (control), BCMA$^{-/-}$ (Xu and Lam, 2001), TACI$^{-/-}$ (Yan et al., 2001b), A/J, and A/WySnJ mice. 10 μg cell lysates were subjected to Western blot analysis with anti-NF-κB2 or anti-β actin.

lymphoid organs, and the inducible processing of the latent transcription factor NF-κB2 (p100) to the active p52 NF-κB subunit (Kaisho et al., 2001; Senftleben et al., 2001). Upon ectopic expression, an IKKα-interacting kinase, NIK, is also capable of triggering NF-κB2/p100 processing (Xiao et al., 2001). Indeed, processing by ectopic NIK is inhibited in IKKα-null lymphoid cells (Senftleben et al., 2001), consistent with IKKα functioning downstream of NIK. In vitro studies suggest that IKKα can directly phosphorylate NF-κB2/p100, leading to ubiquitin-dependent generation of p52 (Senftleben et al., 2001).

The only known activator of the NIK/IKKα/NF-κB2 pathway is the receptor for lymphotoxin-β (LTβR) (Yin et al., 2001). Agonistic LTβR antibodies induce NF-κB2/p100 processing in an NIK- and IKKα-dependent manner (Ghosh and Karin, 2002). LTβR, however, is expressed on stromal cells and not on B lymphocytes, so the B cell ligand/receptor responsible for engaging the NIK/IKKα/NF-κB2 pathway has been enigmatic. BAFF (Moore et al., 1999; Schneider et al., 1999), as a potent survival factor for B cells, is an attractive candidate along with its receptors TACI, BCMA, and BR3/BAFF-R (Gross et al., 2000, 2001; Schiemann et al., 2001; Thompson et al., 2001; Yan et al., 2000, 2001a). Of the three receptors, BR3 is most likely to signal B cell survival, as the BR3/BAFF-R mutant mouse strain A/WySnJ has defects in B cell maturation and lymphoid organ architecture similar to that seen in NIK (Yin et al., 2001)-, IKKα (Kaisho et al., 2001; Senftleben et al., 2001)- and NF-κB2-deficient mice (Caamano et al., 1998; Franzoso et al., 1998). In contrast, BCMA null mice have no discernable phenotype (Schiemann et al., 2001; Xu and Lam, 2001), and TACI null mice p ssess hyperresponsive B cells, splenomegaly, and increased Ig levels (Yan et al., 2001b), consistent with TACI functioning as an inhibitory receptor.

Herein, we show that the BAFF-BR3 interaction does indeed promote processing of NF-κB2/p100, both physiologically and in a murine model of lupus. Additionally, we demonstrate that BR3 binds BAFF through a discrete surface loop; structural mimics of this loop could be used to develop inhibitors to treat autoimmune disorders.

Results and Discussion

BAFF/BR3 Signaling Promotes Processing of NF-κB2

Initially, we determined the impact of BAFF on NF-κB2/p100 processing in the whole animal. As reported previously (Senftleben et al., 2001; Yamada et al., 2000), splenic B cells from untreated, naive mice display basal activation of the NIK/IKKα/NF-κB2 pathway as evidenced by the presence of p52. This signaling presumably results from the presence of an endogenous activator, possibly BAFF, in the splenic microenvironment. To test this possibility, C57BL/6 mice were injected with either purified recombinant TACI-Fc, which neutralizes both BAFF and APRIL, or BR3-Fc, which specifically binds BAFF (Gross et al., 2000; Thompson et al., 2001; Yan et al., 2000, 2001a). In either case, generation of p52 was diminished substantially, implying that endogenous BAFF is required for normal basal processing of NF-κB2/p100 in splenic B cells in vivo (Figure 1A). Nevertheless, BAFF may effect cleavage of NF-κB2/p100 indirectly by inducing other cytokines that activate NF-κB2/p100. To eliminate any contribution fr m accessory cells, splenic B cells purified from mice treated with BR3-Fc (to reduce basal p52 generation) were cultured in the presence of recombinant BAFF. Generation f p52 was augmented significantly in the presence of BAFF, consistent with

Fig. 2a

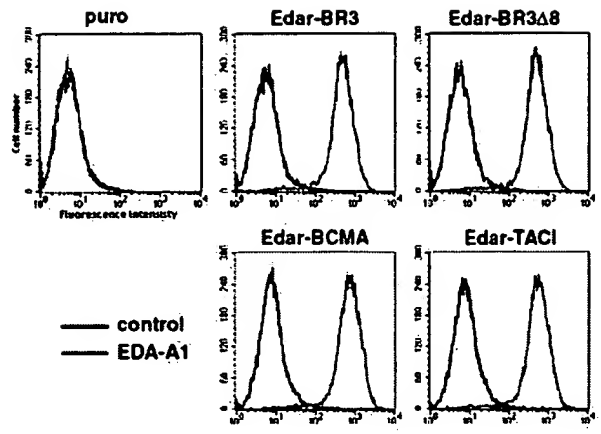


Figure 2. BR3 Activation Is Sufficient for NF-κB2 Processing (p100 to p52)

(A) WEHI 231 murine B lymphoma cells were infected retrovirally with vector alone (puro) or encoding Edar-BR3, Edar-BR3Δ8, Edar-BCMA, and Edar-TACI fusion constructs. The transfectants were stained with FLAG-EDA-A1 followed by anti-FLAG and PE-conjugated anti-rabbit. After washing, cells were analyzed on a FACScan (Becton Dickinson) and data processed using the CELLQuest program (Becton Dickinson).

(B) WEHI 231 transfectants were cultured with 1 μg/ml FLAG-EDA-A1 for 24 hr. 10 μg cell lysates of individual transfectants were subjected to Western blot analysis with anti-NF-κB2 or anti-β actin.

(C) WEHI 231 transfectants were cultured with 1 μg/ml FLAG-EDA-A1 for indicated periods. Cell lysates (10 μg) were subjected to Western blot analysis with anti-phospho-IκB-α and anti-β actin as a loading control.

Fig. 2b

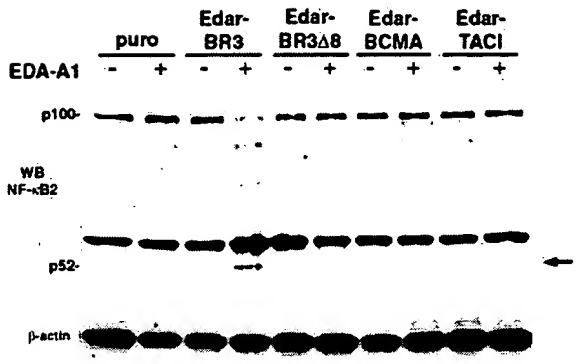
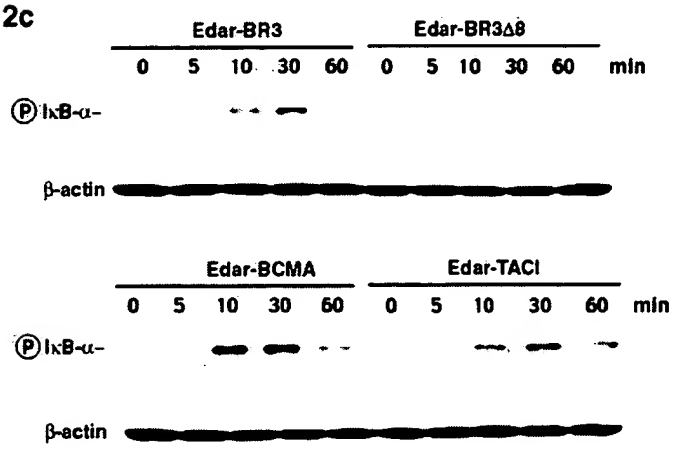


Fig. 2c



BAFF acting directly on B cells to promote NF-κB2 processing (Figure 1B, right 2 lanes). BAFF also increased, to a lesser extent, the amount of p52 in splenic B cells from untreated wild-typ mice (Figure 1B, left 2 lanes), suggesting that endogenous levels of BAFF are not saturating.

Since B cells express all three BAFF receptors (Gross et al., 2000; Schiemann et al., 2001; Thompson et al., 2001; Yan et al., 2000, 2001a), we determined the c ntribution of each to NF-κB2 processing. Similar levels of processed p52 were found in B c lls from control C57BL/6, BCMA-, and TACI-deficient mic (Figure 1C). In sharp contrast, no p52 was detected in B cells from the BR3 mutant A/WySnJ strain. These results c n-

Fig. 3a

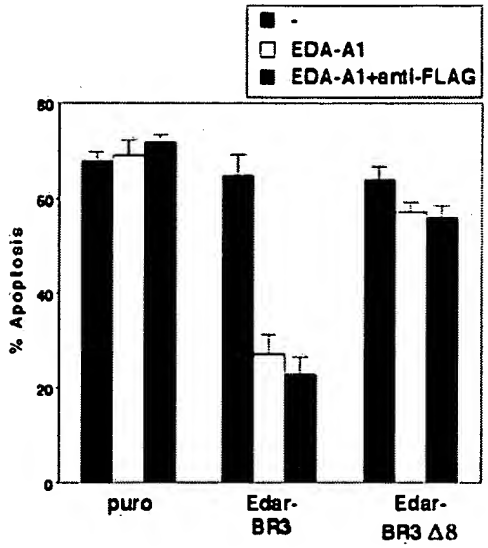


Fig. 3b

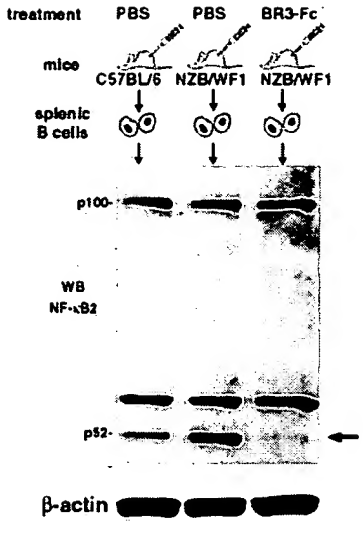


Figure 3. BR3 Crosslinking Can Rescue Anti-IgM-Induced Apoptosis

(A) WEHI 231 transfectants were cultured with or without 1 μg/ml FLAG-EDA-A1 ± 5 μg/ml anti-FLAG Ab for 24 hr followed by coculture with 10 μg/ml anti-IgM for 48 hr. Cells were then stained with FITC-AnnexinV (Becton Dickinson) and analyzed on a FACScan (Becton Dickinson). Data represent the mean ± SD of triplicate samples.

(B) Five 24-week-old NZB/WF1 mice were injected intraperitoneally with PBS or 100 μg BR3-Fc fusion protein three times a week for 5 weeks. 10 μg cell lysates from pooled splenic B cells of 54-week-old mice were subjected to Western blot analysis with anti-NF-κB2 or anti-β actin. Age-matched C57BL/6 mice were used as control.

firmed a critical role for BR3 in BAFF-induced processing of NF-κB2 in B cells.

To extend these in vivo observations, we determined the ability of individual BAFF receptors to induce NF-κB2 processing in vitro. A receptor chimera system was used to stimulate each receptor specifically and to avoid activating endogenous BAFF receptors. Thus, the extracellular ligand binding domains of BCMA, TACI, and BR3 were replaced by the extracellular domain of the ectodysplasin receptor (Edar), a TNF receptor family member expressed in developing skin but not in lymphoid or hematopoietic tissues. Retroviral vectors were used to express Edar-BCMA, Edar-TACI, and Edar-BR3 in the murine B cell line WEHI 231. Expression of the chimeric receptors was confirmed by flow cytometric analysis of cells surface stained with the cognate ligand for Edar, EDA-A1 (Figure 2A). Transfectants were cultured in the presence of EDA-A1 to crosslink and activate the chimeric receptors and then examined for the presence of p52. As shown in Figure 2B, control puromycin-resistant cells only expressed unprocessed NF-κB2/p100. In contrast, p52 was induced in Edar-BR3 transfectants treated with EDA-A1. In keeping with the in vivo data (Figure 1C), p52 was not generated in either Edar-BCMA or Edar-TACI transfectants, despite prolonged culture with EDA-A1 (Figure 2B). Taken together, these data suggest that BR3 alone can induce proteolytic maturation of NF-κB2 to p52. To mimic the insertional mutation that disrupts the eight C-terminal amino acids of BR3 in A/WySnJ mic (Yan et al., 2001a), we generated an Edar receptor chimera lacking these eight residues (Edar-BR3Δ8). This mutant receptor chimera failed to initiate p100 processing following EDA-A1 treatment (Figure 2B), suggesting that the BR3 C terminus is critical for NF-κB2 activation.

Most TNFR members can also activate the classical NF-κB pathway (Ghosh and Karin, 2002). In this pathway, IKKβ phosphorylates IκB, resulting in ubiquitin-dependent degradation of IκB and subsequent translocation of the cytoplasmic p50/p65 transcriptional complex to the nucleus. To determine if any of the BAFF receptors engaged this pathway, we examined the ability of individual BAFF receptors to induce phosphorylation of IκB in WEHI231 transfectants. As shown in Figure 2C, all three BAFF receptors induced phospho-IκB (Ser32/36) within 10 min of activation. BR3Δ8 was unable to induce IκB phosphorylation, suggesting that the C-terminal eight amino acid residues are important for engaging both the classical NF-κB and the NF-κB2 pathways. While these data indicate that all three BAFF receptors can activate the classical NF-κB pathway, the physiological significance of this is still unclear.

BR3 Signaling Protects WEHI 231 Cells from Anti-IgM-Induced Apoptosis

Potentially harmful, autoreactiv B cells are likely kept in check by multiple mechanisms including deleti n (Goodnow, 1992; Hertz and Nemazee, 1998; Klinman, 1996), but inappropriately high levels of a B cell survival factor, such as BAFF, may overcome such regulatory

Fig. 4a

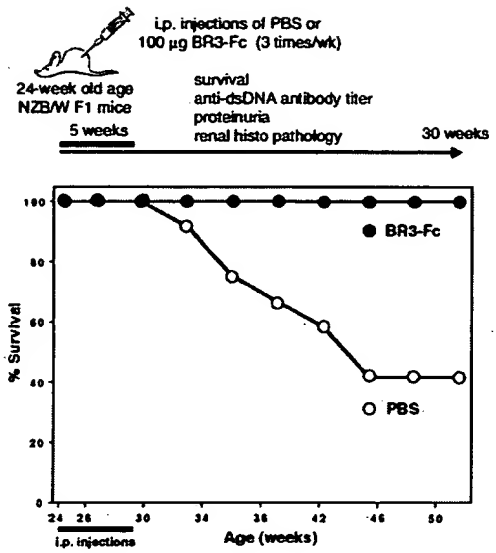


Fig. 4c

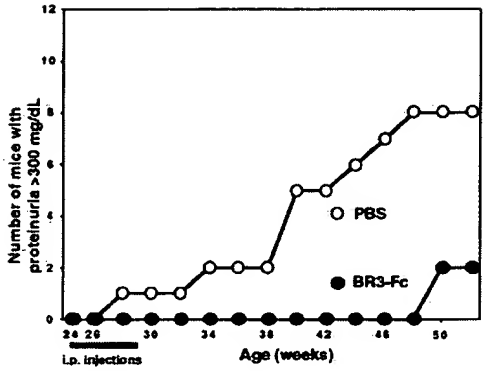


Fig. 4b

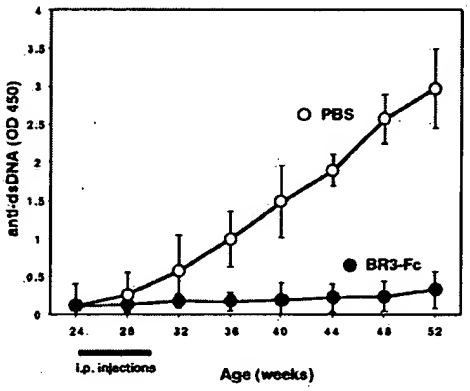


Fig. 4d

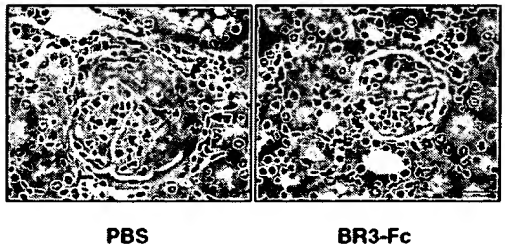


Figure 4. BR3-Fc Blocks Development of Lupus Disease in NZB/WF1 Mice

(A) 24-week-old female NZB/WF1 mice (12 mice per group) were injected intraperitoneally with PBS (open circles) or 100 μg BR3-Fc (filled circles) three times a week for 5 weeks. Mice were checked three times a week for morbidity. The number of mice surviving in each treatment group is shown.

(B) Serum levels of circulating anti-dsDNA antibodies were checked monthly and are shown as OD_{450} values from an ELISA.

(C) Proteinuria was checked monthly and the percentage of mice in each group with levels greater than 300 mg/dL is shown.

(D) 54-week-old NZB/WF1 kidneys from both treatment groups. Arrows indicate fibrinoid necrosis and sclerosis prominent in affected glomeruli accompanied by occasional crescent formation within the PBS-treated mice (left panel) compared to minimal glomerular changes in BR3-Fc-treated animals (right panel).

mechanisms. Indeed, elevated BAFF serum levels have been reported for patients with SLE (Zhang et al., 2001) and Sjögren's syndrome (Groom et al., 2002), although a causal link is yet to be established. An in vitro model for B cell negative selection is the induction of apoptosis in WEHI 231 cells by crosslinking anti-IgM antibody (Benhamou et al., 1990; Hasbold and Klaus, 1990). We found that prior engagement of Edar-BR3 with either FLAG-EDA-A1 alone or FLAG-tagged ligand hyper-crosslinked with anti-FLAG antibody caused a marked reduction in anti-IgM-induced apoptosis. In contrast, crosslinking Edar-BR3Δ8 failed to rescue the anti-IgM stimulated cells (Figure 3A). Thus, the C terminus of BR3 is critical for p52 generation, phospho-IκB induction, and th pro-survival role of BR3 in B cells.

Blockade of BAFF Signaling in NZB/WF1 Mice Abrogates p52 Generation and the Development of Spontaneous Lupus-like Disease

Given that BR3 signaling can protect WEHI 231 cells from apoptosis induced by engagement of the B cell receptor, inappropriate activation of the BAFF-BR3 axis might contribute to the emergence of autoreactive B cell clones that play a pathogenic role in autoimmune disease. To investigate this possibility, we studied NZB/WF1 mice that develop spontaneous, fatal lupus-like disease. At 22–24 weeks of age, NZB/WF1 mice exhibited a slight but reproducible increase in the level of p52 in their B cells when compared to C57BL/6 mice (Figur 3B). This finding is consistent with the elevated levels of BAFF in NZB/WF1 mice (Gross et al., 2000). To assess

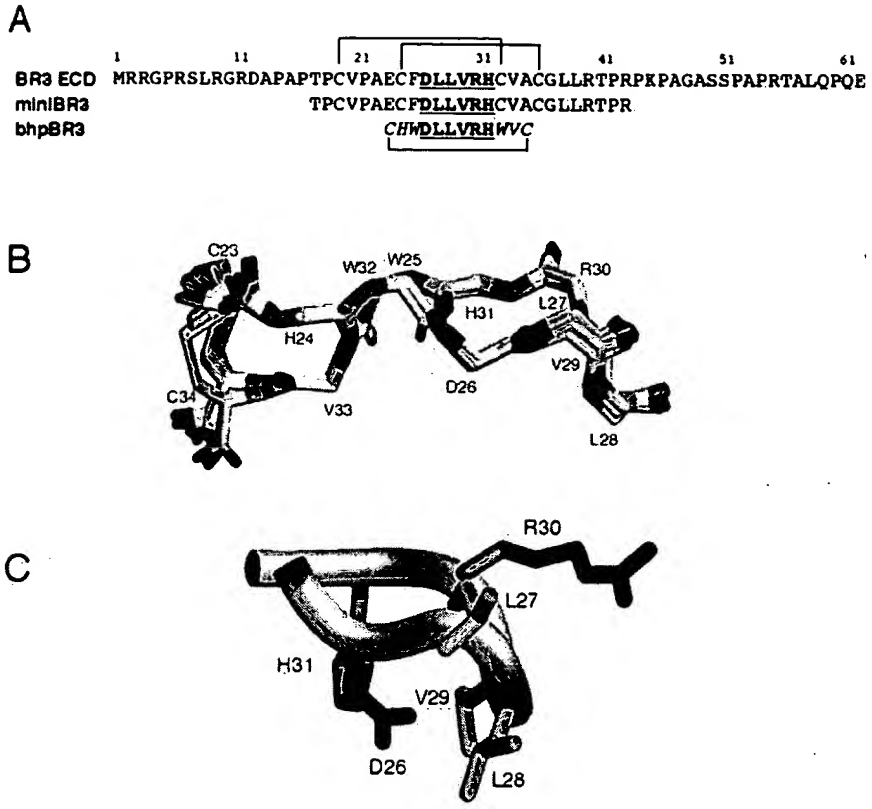


Figure 5. Sequences of BR3 Variants and Structure of bhpBR3

(A) Amino acid sequences of BR3 variants used in this study.

(B and C) Three-dimensional structure of bhpBR3 determined by NMR spectroscopy. The backbone atoms of 20 models are shown superposed with residue labels positioned in the direction of the side chain (B); one representative structure highlighting the BR3 turn residues (C) in the same orientation as in B.

directly the contribution of BAFF to disease progression in NZB/WF1, we treated 24-week-old female animals (12 per group) with a short 5 week course of BR3-Fc. This treatment dramatically attenuated the lupus-like disease for the remaining 30 week length of the study: B cell p52 levels were markedly reduced (Figure 3B), there was a 100% survival rate (Figure 4A), and the mice possessed fewer anti-double-stranded (ds) DNA antibodies (Figure 4B). Renal damage was also less severe as evidenced by diminished proteinuria (Figure 4C) and only mild glomerular changes on renal histology (Figure 4D). Previous studies have shown that treatment with TACI-Fc, which binds both BAFF and APRIL, also suppresses proteinuria but does not block the generation of anti-dsDNA antibodies (Gross et al., 2000). It is not clear why BR3-Fc, which exclusively binds BAFF (Thompson et al., 2001; Yan et al., 2001a), should so potently suppress autoantibody titers, but it may relate to pharmacokinetic differences or a greater potency in neutralizing bioavailable BAFF.

To assess the effect of initial BR3-Fc treatment on long-term leukocyte counts, peripheral blood was analyzed at the time of sacrifice. Leukocyte counts including B cell numbers were within normal limits at the end of the study. Thus, the initial BR3-Fc treatment may have eliminated or silenced B cell populations that play a pathogenic role in the development of this autoimmune disorder. In contrast, control-treated animals rapidly developed anti-dsDNA autoantibodies (Figure 4B) and succumbed to diffuse membranoproliferative glomerulonephritis characterized by mesangial proliferation and inflammatory cell infiltrates. Fibrinoid necrosis and sclerosis were prominent in affected glomeruli accompanied by occasional crescent formation (Figure 4D). In sum, the data suggest that preventing the BAFF-BR3 interaction may represent a viable therapeutic treatment for lupus.

The BAFF Binding Site within BR3 Is Contained within a 26 Residue Core Domain

As a first step toward developing agents to disrupt the BAFF-BR3 interaction, an NMR analysis of the extracellular ligand binding domain of BR3 was performed. Intriguingly, only the central one-third of the protein adopts a stable structure in solution; this core is stabilized by two disulfide bonds connecting Cys19/Cys32 and Cys24/Cys35 (M.A.S., J.Y., and W.J. Fairbrother, unpublished data). C nsequently, a 26 residu miniBR3 peptide was synthesized, incorporating th 1:3, 2:4 disulfide-bonding pattern, and characterized structurally (Figure 5A). Indeed, NMR spectra of miniBR3 indicated that this peptide adopts essentially the same structure

Fig. 6a

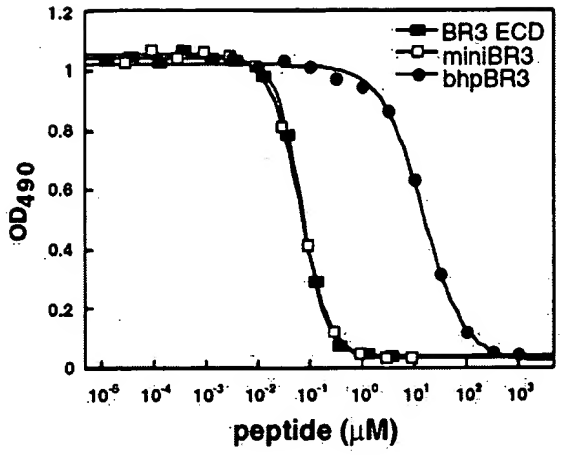


Fig. 6b

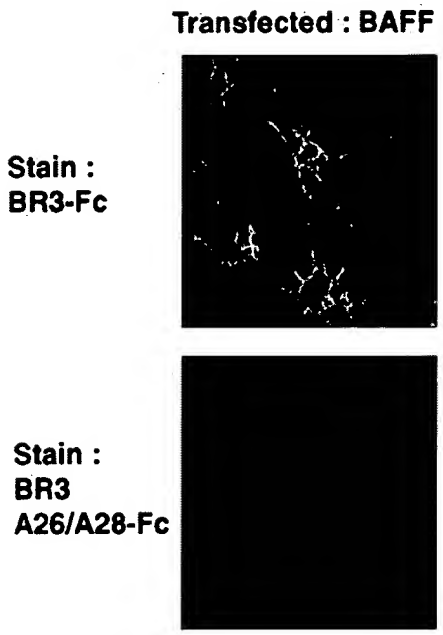


Fig. 6c

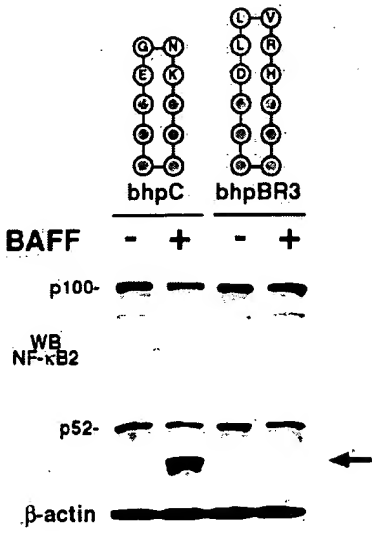


Figure 6. Binding of BR3 Variants to BAFF

(A) Competitive displacement of biotinylated miniBR3 measured by ELISA (see Experimental Procedures). Data are shown for BR3 extracellular domain (filled squares), miniBR3 (open squares), and bhpBR3 (filled circles). IC_{50} values from the fitted curves are 70 nM, 65 nM, and 15 μM, respectively.

(B) COS 7 cells transfected with BAFF were stained with BR3-Fc or BR3 A26/A28-Fc followed by Cy3-labeled anti-human IgG Fc.

(C) Splenic B cells from BR3-Fc-treated mice were cultured for 24 hr in the presence or absence of recombinant BAFF (2 nM), which had been preincubated for 30 min with bhpBR3 (60 μM) or a control hairpin peptide composed of an unrelated turn sequence in the same bhp scaffold (bhpC, 60 μM). Thereafter, B cell lysates (10 μg) were subjected to Western blot analysis with anti-NF-κB2 or anti-β actin.

as in the context of the full-length protein (not shown). Importantly, miniBR3 also binds with the same affinity as full-length BR3 to BAFF (~70 nM IC_{50}; Figure 6A).

Given that high-affinity BAFF binding was contained within a 26 residue core, we attempted to further delineate the BAFF binding p rtion of BR3. In crystal structures of other TNF-like ligand/recept r c mplexes, a receptor loop analogous to BR3 residues ^{26}DLLVRH^{31} is involved in forming direct c ntacts with the ligand (Bodmer et al., 2002, and references therein). Intrigu-

ingly, all three BAFF receptors share the short motif "DxL" at the beginning of this loop (see below). The importance of the conserved motif for BAFF binding was tested by site-directed mutagenesis. As shown in Figure 6B, replacement of the two invariant loop residues of BR3-Fc (D26 and L28) with alanine resulted in complete loss of binding to BAFF, confirming the importance of the loop in BAFF recognition.

In the context of both full-length and miniBR3, NMR analysis suggested that the critical loop sequence ^{26}DLLVRH^{31} presents a type I β turn centered at L28/V29 with potential for backbone hydrogen-bonding between Asp26 and His31. Thus, in order to test whether this loop might be sufficient for binding, we synthesized a 12 residue peptide in which the six residues from BR3 were embedded within a disulfide-bonded β hairpin (bhp) scaffold (Figure 5A). Previously, we have shown that the strong strand-strand interactions in these scaffolds can structure a variety of β turns (Cochran et al., 2001; S.J. Russell, T. Blandl, N.J. Skelton, and A.G.C., unpublished data; Russell and Cochran, 2000).

The peptide bhpBR3 adopts a remarkably stable conformation in solution as indicated by a high degree of chemical shift dispersion, extreme values for many of the backbone and side chain coupling constants, and a large number of long-range NOEs present in its NMR spectra (see supplemental table S1 at http://www.immunity.com/cgi/content/full/17/4/515/DC1). The three-dimensional structure of bhpBR3 consists of a β hairpin in which the BR3 turn sequence adopts the type I β turn structure, as expected, with Arg30 adopting a positive ϕ angle and the side chains of the invariant Asp26 and Leu28, as well as those of Val29 and His31, projecting on one face of the β turn (Figures 5B and 5C). If BR3 binds BAFF using interactions homologous to those observed for TNFR and DR5 (Hymowitz et al., 1999), then one would expect this face (the "bottom" face of the turn shown in Figure 5C) to contact BAFF.

Because bhpBR3 structurally mimics the BR3 turn, we tested whether it could compete with miniBR3 for binding to BAFF (Figure 6A). Remarkably, the 12 residue peptide blocked binding of the larger core domain (IC_{50} = 15 μM), indicating that the critical binding determinants do indeed reside in the six residue loop shown in Figure 5C. Finally, we tested whether bhpBR3 could function in a bioassay: bhpBR3, but not a control hairpin peptide, blocked BAFF-mediated NF-κB2/p52 induction in primary B cells (Figure 6C).

Our finding that a β turn structure from BR3 has significant affinity for BAFF has implications for recognition of BAFF by its other receptors. TACI and BCMA share homologous sequences in this loop region that would be expected to adopt a similar turn conformation to that in BR3 (DSLLHA, DPLLGT, and DHLLRD, for BCMA and CRDs 1 and 2 of TACI, respectively). Therefore, the interactions of this turn with ligand will likely be a conserved feature of all BAFF/receptor complexes. The surprising identification of such a focused recognition epitope will provide the framework for developing small-molecule peptidomimetic inhibit rs of the BAFF-BR3 interaction; these inhibitors may have therapeutic potential in the treatment of autoimmune diseases such as lupus.

Experimental Procedures

Isolation and Culture of B Cells

Splenic B cells were isolated using MACS beads (Miltenyi). Recovered cells were >96% $B220^+IgM^+$ B cells. In some experiments, freshly isolated B cells were further cultured with 1 μg/ml recombinant BAFF (Yan et al., 2001a) for 24 hr.

Western Blot Analysis

Cell lysis and immunoblotting were performed as described (Humke et al., 2000). Anti-NF-κB2 (Santa Cruz) was used to detect p100 and p52. Phospho-IκB-α-specific antibody (Ser32/36, Cell Signaling) was used to detect phospho-IκB-α.

Retroviral Construction and Infection

Extracellular domains of BCMA (1-42), TACI (1-114), and BR3 (1-55) were replaced by Edar (1-182) by recombinant PCR. Resulting cDNAs were subcloned into a puromycin-selectable retrovirus vector. Production and infection of retrovirus were performed as described elsewhere (Humke et al., 2000). WEHI 231 cells stably expressing chimeric receptors were selected using 1 μg/ml puromycin.

NZB/WF1 Lupus Mice Studies

Female NZB/WF1 mice were purchased from Jackson. Proteinuria levels were monitored by Uristix (Ames). Anti-dsDNA antibody titers in the serum were measured using poly L-lysine/poly dAdT (Sigma) coated plates and detected with HRP-conjugated goat anti-mouse IgG antibody. In some experiments, kidneys from 54-week old NZB/WF1 mice were fixed in 10% neutral-buffered formalin and embedded in paraffin. Three micron thick sections stained with hematoxylin and eosin were examined by light microscopy.

Protein Production

The extracellular domain of BR3 was subcloned into the pET32a expression vector (Novagen), creating a fusion with an N-terminal thioredoxin (TRX)-His-tag followed by an enterokinase protease site. *E. coli* BL21(DE3) cells (Novagen) were grown at 30°C and protein expression was induced with IPTG. TRX-BR3 was purified over an Ni-NTA column (Qiagen), eluted with an imidazole gradient, and cleaved with enterokinase (Novagen). BR3 was then purified over an S-Sepharose column, refolded overnight in PBS (pH 7.8), in the presence of 3 mM oxidized and 1 mM reduced glutathione, dialyzed against PBS, repurified over a MonoS column, concentrated, and dialyzed into PBS.

Peptide Synthesis

MiniBR3 and bhpBR3 were synthesized as C-terminal amides on a Pioneer peptide synthesizer (PE Biosystems) using standard Fmoc chemistry. For miniBR3, the side chain thiols of cysteines 19 and 32 were protected as the trifluoroacetic acid (TFA)-stable acetamidomethyl (Acm) derivatives. Peptides were cleaved from resin by treatment with 5% triisopropyl silane in TFA for 1.5–4 hr at room temperature. After removal of TFA by rotary evaporation, peptides were precipitated by addition of ethyl ether, then purified by reversed-phase HPLC (acetonitrile/H_2O/0.1% TFA). Peptide identity was confirmed by electrospray mass spectrometry. BhpBR3 was converted to the cyclic disulfide by dropwise addition of a saturated solution of I_2 (in acetic acid) to HPLC fractions. After lyophilization, the oxidized peptide was purified by HPLC. HPLC fractions containing reduced miniBR3 were adjusted to a pH of ~9 with NH_4OH; the disulfide between cysteines 24 and 35 was then formed by addition of a small excess of $K_3Fe(CN)_6$, and the oxidized peptide purified by HPLC. Acm groups were removed (with concomitant formation of the second disulfide) by treatment of the HPLC eluate with a small excess of I_2 over ~4 hr. The progress of the oxidation was monitored by analytical HPLC, and the final product was again purified by HPLC. MiniBR3 was amino-terminally biotinylated while on resin, then cleaved and purified exactly as described above for the unmodified peptide.

NMR Spectroscopy

Two-dimensional (2D) NMR experiments were acquired and analyzed as described (Starovasnik et al., 1996) using a Bruker DRX-

600 spectrometer at 293K on a sample containing 2.9 mM bhpBR3 (pH 4.5), with 0.1 mM DSS as a chemical shift reference. Distance restraints were derived from 2D NOESY spectra (τ_m 250 ms); dihedral angle restraints were derived from analysis of a 2D DQF-COSY spectrum acquired in 92%H_2O/8%D_2O and a 2D COSY-35 spectrum acquired on a sample dissolved in 100% D_2O. Complete resonance assignments and coupling constant values are included in the supplemental data.

The three-dimensional structure of bhpBR3 was calculated based on 119 NOE-derived (including 46 long-range) distance restraints and 16 dihedral angle restraints. 100 initial structures were calculated using DGII; 80 of these were further refined by restrained molecular dynamics using DISCOVER as described (Starovasnik et al., 1996). Twenty structures having the lowest restraint violation energy represent the solution conformation of bhpBR3. The model with the lowest rms deviation (RMSD) to the average coordinates of the ensemble was chosen as the representative structure (model 1 in the PDB file). The final ensemble of twenty models satisfies the input data well, having no distance or dihedral angle restraint violations greater than 0.1 Å or 1°, respectively. The structures are well defined, with an average backbone RMSD to the mean coordinates of 0.24 ± 0.06 Å, and have good covalent geometry as judged by PROCHECK (86% of the residues in the most favored, 10% in the allowed, and 4% in the generously allowed regions of $\phi\psi$ space) (Laskowski et al., 1993). The structure of bhpBR3 will be available from the RCSB Protein Data Bank (ID code 1MPV).

Competitive Displacement ELISA

Nunc Maxisorp 96-well plates were coated overnight at 4°C with 100 μl of a 2 μg/ml solution of BAFF in carbonate buffer (pH 9.6). The plate was washed with PBS and blocked with 1% skim milk in PBS. Serial dilutions of BR3 variants were prepared in PBS/0.05% Tween 20 containing 3 ng/ml biotinylated miniBR3. After washing with PBS/Tween, 100 μl/well of each dilution was transferred and incubated for 1 hr at room temperature. The plate was washed with PBS/Tween and incubated for 15 min with 100 μl/well of 0.1 U/ml Streptavidin-POD (Boehringer Mannheim) in PBS/Tween. After washing with PBS/Tween followed by PBS, the plate was incubated for 5 min with 100 μl PBS substrate solution containing 0.8 mg/ml OPD (Sigma) and 0.01% H_2O_2. The reaction was quenched with 100 μl/well 1 M H_3PO_4 and the plate read at 490 nm. IC_{50} values were determined by a four-parameter fit of the competitive displacement ELISA signal. The concentrations of initial stock solutions of bhpBR3 were determined spectrophotometrically as described (Gill and von Hippel, 1989), while those of miniBR3 and BR3 extracellular domain were determined by quantitative amino acid analysis.

BR3-Fc Staining of BAFF-Transfected COS7 Cells

Mutations that disrupt residues within the ^{26}DLLVRH^{31} loop (D26→A26 and L28→A28) were introduced into BR3-Fc cDNA (Yan et al., 2001a) by PCR. COS7 cells transfected with BAFF were stained with 1 μg/ml BR3-Fc or BR3 A26/A28-Fc. Cells were washed and fixed, and bound receptor detected with Cy3-labeled anti-human IgG Fc (Jackson).

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Accession Number

The structure of bhpBR3 will be available from the RCSB Protein Data Bank under ID code 1MPV.